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ENDOCRINE EVENTS PRIOR TO PUBERTY IN HEIFERS: ROLE OF SOMATOTROPIN, INSULIN-LIKE GROWTH FACTOR-I AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

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We have utilized active immunization against growth hormone releasing factor (GRF) to investigate relationships among somatotropin (ST), insulin-like growth factor-I (IGF-I), IGF binding proteins (IGFBP) and ovarian function in heifers. Active immunization against GRF (GRFi) has been demonstrated to abolish episodic release of ST and decrease serum concentrations of IGF-I. In initial experiments investigating onset of puberty, breeds of heifers differing in growth rate and reproductive traits (Angus, Charolais and Simmental) were immunized against GRF or served as controls (immunized against carrier protein, human serum albumin, HSAi). GRFi decreased rate of muscle and skeletal growth, but increased deposition of adipose tissue. In Angus and Charolais, but not Simmental heifers, GRFi at 6 mo of age significantly delayed onset of puberty beyond 18 mo of age. Retrospective analyses of serum IGF-I revealed that GRFi heifers reaching puberty at a normal age had greater pre-treatment (6 mo of age) IGF-I than GRFi heifers in which puberty was delayed. Collectively, these results strongly indicate that the bovine hypothalamic-hypophyseal-ovarian axis is particularly sensitive to changes in metabolism at or near 6 mo of age.

Another series of experiments tested the hypothesis that lowering serum IGF-I via GRFi initially at 3 mo of age would increase the percentage of Angus and Simmental heifers not reaching puberty. Three mo old Angus and Simmental heifers were assigned to GRFi (n = 18), HSAi (n = 14) or received no treatment (controls, n = 16). HSAi and GRFi heifers were unilaterally ovariectomized (ULO) at 6 mo of age. As anticipated, GRFi at a younger age increased percentage of heifers not reaching puberty; over 75% of control and HSAi heifers reached puberty by 14 mo of age compared to 22% of GRFi heifers. Serum and follicular fluid (FFL; follicles $\leq$4 mm) concentrations of IGF-I were suppressed by GRFi. Serum, but not FFL concentrations of IGF binding protein-2 (IGFBP-2) were greater in GRFi than in HSAi heifers. GRFi delayed puberty apparently by suppressing follicular growth because number of follicles $\leq$7 mm was significantly

lower in GRFi than in HSAi heifers. In conclusion, active immunization against GRF at 3 or 6 months of age delays puberty in beef heifers. Delayed puberty was preceded by suppression of follicular growth, and decreased concentrations of IGF-I in serum and follicular fluid. Collectively, these studies demonstrate that ovarian function between 3 and 8 mo of age and subsequent onset of puberty are particularly sensitive to changes in the ST-IGF-I axis.

Key words: *heifer, IGF-I, GRF immunization, follicle, IGF binding proteins, puberty*

INTRODUCTION

Lifetime productivity of beef females is enhanced if heifers conceive at 13 mo of age (1). Several factors such as breed, nutritional status, and exposure to bulls influence timing of puberty (2). Of most practical importance is the effect of nutritional status. Restriction of feed intake, either experimentally- or environmentally-induced, delays puberty in heifers (2,3). A better understanding of mechanisms through which alterations in metabolism affect ovarian function and ultimately puberty could lead to management practices that would increase overall productivity.

Changes in the hypothalamo-hypophyseal-ovarian axis prior to puberty have been previously reported (2,3). Puberty in heifers is preceded by an increase in frequency of episodic release of luteinizing hormone (LH), presumably due to a decrease in negative feedback of estradiol on LH (4-7). The anterior pituitary and ovary are responsive to gonadotropin releasing hormone (GnRH) and gonadotropins, respectively, as early as 3 months of age (8-11). However, induction of follicular growth and ovulation in prepuberal heifers is typically not followed by normal estrous cycles (9). Thus, changes in the hypothalamus, particularly sensitivity to negative feedback effects of estradiol and alterations in frequency of GnRH release, are prerequisite for normal timing of puberty in the heifer.

Several researchers have demonstrated that feed restriction affects ovarian function through a decrease in frequency of LH release (12-14). Potential signals mediating effects of a nutritional stress on ovarian function have been the subject of several reviews (13-16). However, precise signals through which alterations in metabolism affect hypothalamo-hypophyseal-ovarian function have not been elucidated. Experiments conducted in several species have demonstrated that restriction of feed intake elicits changes in metabolic hormones and metabolites. For example, concentrations of non-esterified fatty acids increase and insulin decrease (12,17,18) during feed restriction. In addition, feed restriction alters the ST, IGF-I and IGFBP axis (19-21). The normal positive relationship between ST and IGF-I is "uncoupled" by restriction of feed intake; concentrations of ST increase and IGF-I decrease after feed restriction in ruminants (22-24). Decreased serum IGF-I and

decreased responsiveness of IGF-I to ST are temporally related to a reduction in hepatic ST receptors (25). Studies have demonstrated that feed restriction causes a decrease in hepatic concentration of IGF-I mRNA (26,27); however, restriction of protein intake may alter stability of 7.5-kb IGF-I mRNA (28).

In addition to "uncoupling" ST and IGF-I, feed restriction causes shifts in abundance of serum IGFBP (19-21). In particular IGFBP-1 has also been demonstrated to be involved in plasma glucose regulation (29,30). Evidence for metabolic regulation of IGFBP has also been demonstrated using the diabetic pig model. Diabetes causes a decrease in serum IGF-I (31) and a decrease in a 29 kDa IGFBP (32). More recently, Hughey et al. found that FFL from diabetic pig follicles had lower IGFBP activity than non-diabetic pig follicles (33). Less data is available for effects of nutrition on IGFBP in ruminants (21). One report found that feed restriction in sheep was associated with a decrease in serum IGFBP-3 (34).

Much information is available with regard to effects of IGF-I and IGFBP on ovarian function *in vitro*. Several reports have demonstrated effects of IGF-I and IGFBP on granulosa cell differentiation and proliferation (35-37). *In vitro,* IGF-I enhances FSH-stimulated steroid production in murine (35) and porcine (36,37) granulosa cells. Addition of FSH increases IGF-I (36) and decreases IGFBP secretion (37,38) by porcine granulosa cells. IGFBP may serve as additional regulators of FSH-stimulated steroid production by granulosa cells and follicular differentiation. Several IGFBP mRNA are found in rat (39) and pig (38,40) ovaries. In both rat (41) and pig (38) granulosa cell cultures, low doses stimulate and high doses of FSH inhibit IGFBP activity. Intra-ovarian IGFBP are further regulated by IGF-I. Grimes and Hammond (42) recently reported that IGF-I stimulated production of IGFBP-2 and -3 by porcine granulosa cells *in vitro*.

Less information is available with regard to *in vivo* effects of IGF-I and ST on ovarian function. *In vivo* effects of ST on ovarian function have been studied in cattle (43-48) and gilts (49-53) and have yielded equivocal effects. More recent reports have demonstrated that ST *via* direct or indirect effects may alter folliculogenesis. Exogenous ST administered to lactating cows (54,55) or non-lactating heifers (44) increased number of medium sized follicles, whereas ST increased number of small follicles in gilts (53). In addition, in two separate reports ST increased serum progesterone (43,48); however, effects on LH and FSH were equivocal (43,48). A positive effect of ST and(or) IGF-I on follicular growth is consistent with reports in humans that concomitant administration of ST enhances effectiveness of gonadotropins for induction of ovulation (56).

Putative mechanisms through which ST alter follicular growth include direct effects on the ovary, indirect *via* the hypothalamo-hypophyseal axis and(or) indirect through IGF-I at any or all of the aforementioned sites. Lucy

(57) reported that ST receptors are present in the bovine ovary, with concentrations of ST receptor mRNA and receptors greater in the CL than in follicles (57). Thus, ST may alter ovarian function *via* direct effects on the ovary with IGF-I likely acting as an intra-ovarian mediator. However, an endocrine effect of IGF-I cannot be excluded (58,59).

In addition to IGF-I receptors and mRNA present in the ovary (35,36) and liver (26), IGF-I mRNA and receptors are present in the median eminence (60-62). A recent report adds credence to possible endocrine effects of IGF-I; *in vitro* release of GnRH by rat median eminence was increased by IGF-I (63). Both IGF-II and insulin were effective but at greater concentrations.

The GRF-ST-IGF-I axis could also affect ovarian function *via* direct effects of peripherally or locally produced GRF. Spicer and Enright (64) found that exogenous GRF altered number of medium follicles in heifers. Several reports have demonstrated that GRF affects granulosa cell function *in vitro* (65-67). Finally, GRF mRNA is present in the rat ovary (68). Further studies are needed to clarify mechanisms through which alterations in GRF-ST-IGF-I alter ovarian function.

Based on the aforementioned effects of ST and IGF-I on ovarian function *in vitro* and *in vivo*, we felt it was particularly important to develop an *in vivo* model in which we could alter peripheral and tissue ST and IGF-I concentrations. For our model we have utilized active immunization against GRF conjugated to human serum albumin (GRFi). GRFi effectively lowers serum ST by inhibiting episodic release from the anterior pituitary. Consequently, serum ST and IGF-I concentrations are greatly diminished but remain detectable. We have utilized GRFi to investigate relationships among metabolism, body composition and reproduction in porcine (69,70) and bovine (71,72) species under a variety of physiological states. The remainder of this paper will focus on our studies investigating relationships among ST, IGF-I, IGFBP, growth and puberty in heifers.

Effect of GRFi on ST, IGF-I and IGFBP in serum

In initial studies, we actively immunized gestating-lactating females (69,71) or postpuberal females (70) against GRF. GRFi abolished episodic release of ST in bovine (heifers and cows) and porcine females (gilts). In addition, serum concentrations of IGF-I were significantly decreased (70,71). More recently, we evaluated effects of GRFi on hormones and metabolites in heifers (72) and steers (73). Both frequency of release and mean ST were decreased by GRFi.

Active immunization against GRF, in addition to producing the aforementioned effects on ST and IGF-I, alters other metabolites and hormones. Serum concentrations of insulin and to a lesser extent, glucose are consistently decreased by GRFi in heifers (72). In contrast, we have failed to

observe an effect of GRFi on serum NEFA. These changes in metabolism are consistent with previously reported effects of exogenous ST on insulin, glucose and body composition in cattle (74,75) and swine (76). In addition, we previously reported that GRFi during gestation and lactation in pigs increased fat deposition *in vivo* and lipogenesis *in vitro* (77). Consistent with these results in swine, GRFi increased deposition of fat in heifers (72) and steers (78).

Based on previous results (19,21), we hypothesized that GRFi and the resulting changes in ST and IGF-I would drastically alter profiles of serum IGFBP (i.e. — IGFBP-3 would decrease; IGFBP-2 would increase). As anticipated, serum concentrations of IGFBP-3 were decreased by GRFi. This was likely due to decreased IGF-I and(or) ST (19,21). Serum concentrations (ng/ml) of IGFBP-2 were consistently greater in GRFi (594 $\pm$48) than in HSAi (384$\pm$52) heifers (79). These effects on IGFBP-2 and -3 were reversed by administration of exogenous ST (79). These observations are consistent with previous reports by Cohick et al. (80) and others (21,81). Thus, GRFi provides an excellent model for altering concentrations of ST, IGF-I and IGFBP *in vivo*.

Effect of GRFi on prepuberal ovarian function and puberty in heifers

We reasoned that GRFi at an early age would alter growth rate and body composition and thus provide a model to investigate relationships among body composition, metabolism and onset of puberty in heifers. In our initial experiment (72), we evaluated effects of GRFi in two diverse genotypes (Angus and Charolais) differing in age at puberty, mature body weight and body composition. We actively immunized Angus and Charolais (n=43) heifers against GRF or HSA at 6 months of age. Boosters of appropriate antigen were given at 7.5 mo of age and at 2-3 mo intervals thereafter. GRFi decreased deposition of muscle and skeletal development; however, fat depth was increased. These changes were associated with decreased ST and IGF-I. Effects of GRFi on body composition, ST and IGF-I were similar in Angus and Charolais heifers (72).

Our most exciting result was the effect of GRFi on puberty; GRFi delayed puberty beyond 18 mo in over 40% of heifers (*Fig. 1*) (72). In addition, treatment effects were similar in Angus and Charolais heifers. Differences in average concentrations or frequency of release of LH were not detected. As depicted in *Fig. 1.*, effects of GRFi on puberty were an "all or none" phenomena, i.e. heifers reached puberty by 12-13 mo of age or they remained acyclic until at least 18 mo of age (82). We were therefore interested in ascertaining potential differences between heifers that cycled or remained acyclic. We found that serum concentrations of IGF-I at time of treatment (6 mo of age) and average daily gain from 6 to 8 mo of age were greater in GRFi heifers that reached puberty than in GRFi heifers that were acyclic at 18

mo of age (72). We were interested in determining whether antibody production differed in cyclic versus acyclic heifers. Percentage binding of ^{125}I-GRF one week after first booster was greater in acyclic than in cyclic Angus heifers; however, percentage binding was not related to occurrence of puberty in Charolais heifers (72).

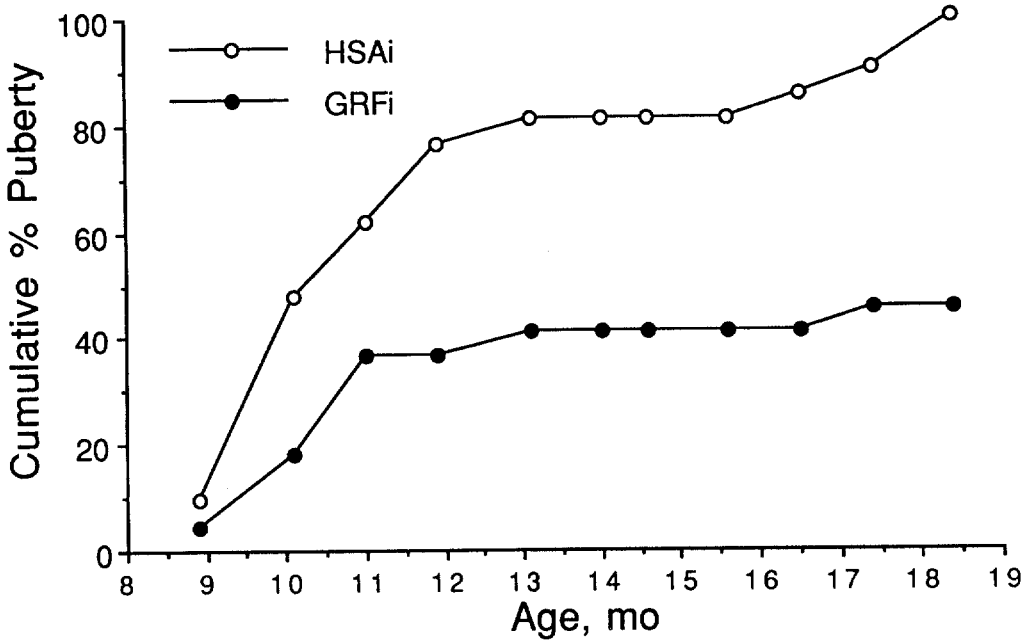

Fig. 1. Cumulative percentage heifers attaining puberty from 8 to 19 mo of age in Angus and Charolais heifers immunized at 6 mo of age against HSA or GRF. Onset of puberty was similar in Angus and Charolais heifers thus data were pooled. Significantly fewer GRFi heifers reached puberty by 18 mo of age compared to controls (HSAi) (72).

The "all or none" effect of GRFi on puberty has provided an excellent opportunity to evaluate effects of low serum IGF-I and ST on cycle length, serum progesterone and conception rates. Initially, we characterized concentrations of progesterone and IGF-I in cyclic, control (HSAi) and GRFi heifers. Serum samples were collected every other day *via* venipuncture from HSAi (n = 14) and GRFi (n = 9) heifers. Length of estrous cycle or luteal phase (based on serum progesterone) was not affected by GrFi. Furthermore, concentrations of progesterone were similar in GRFi and HSAi heifers. Serum IGF-I did not vary significantly with day of cycle in either HSAi or GRFi heifers. Finally, all heifers were exposed to bulls, conceived and calved. Thus, providing heifers attain puberty, GRFi did not alter progesterone, cycle length or ability of heifers to become pregnant and calve.

Our next study evaluated the hypothesis that GRFi delays puberty independent of effects of GRFi on ADG (83). Angus, Charolais and Simmental heifers were assigned to GRFi or HSAi at 6 mo of age. At 7.5 mo

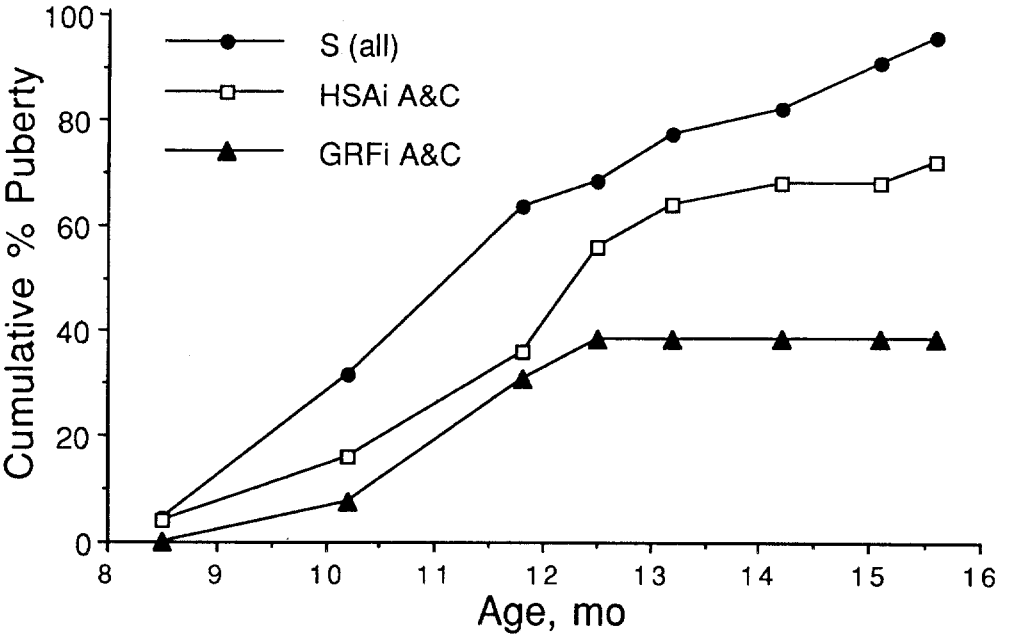

Fig. 2. Onset of puberty in GRFi and HSAi Simmental (S), Angus (A) and Charolais (c) heifers. Pubertal onset was not affected by GRFi or feed restriction in S heifers; therefore, data were pooled and represented as S (all). Feed restriction failed to alter timing of puberty in A and C heifers, thus data were pooled (HSAi A & C). Immunization against GRF delayed puberty in A and C, but not in S heifers (83).

of age, HSAi heifers were fed to gain .9 (HSAi-C) or restricted in feed intake to gain .7 (HSAi-R) kg/d. GRFi heifers were fed to gain .9 kg/d with an expected gain of .7 kg/d. Puberty was monitored by weekly serum progesterone concentrations and frequent blood samples (every 15 min for 8 h) were collected at 3 wk intervals from 28 heifers. As anticipated , ADG and serum IGF-I were lower in GRFi and HSAi-R than in control heifers. However, concentrations of IGF-I were only marginally decreased by HSAi-R, particularly when compared to effects of GRFi. Further analyses revealed that serum LH was decreased by HSAi-R, but not by GRFi. Although feed restriction had marginal yet significant effects on IGF-I and LH, onset of puberty was not affected in Angus, Charolais and Simmental heifers. Consistent with our previous report, GRFi delayed puberty ($P<.05$) in Angus and Charolais heifers (*Fig. 2*). In contrast, percentage of heifers puberal by 15 mo of age was similar in GRFi and control Simmental heifers (*Fig. 2*). These results indicated a breed difference in mechanism or timing of mechanism with regard to effects of IGF-I on prepuberal ovarian function. Collectively, these data indicate that GRFi results in greater reductions in IGF-I than feed restriction. In addition, absence of effects of GRFi on LH indicate a potential ovarian site of action.

Based on previous results suggesting that onset of puberty in GRFi heifers was related to IGF-I at time of treatment (6 mo of age) (72) and GRFi at 6 mo

of age failed to delay puberty in Simmental heifers (83), we hypothesized that GRFi at an earlier age would delay puberty in a greater percentage of heifers. To test this hypothesis we assigned Angus and Simmental heifers to GRFi, HSAi or no treatment (Con) at 3 mo of age (84). In addition, we unilaterally ovariectomized (ULO; left ovary) GRFi and HSAi heifers at 6 mo of age in order to determine effects on follicular growth. Follicular diameters were recorded and follicular fluid aspirated. As hypothesized, GRFi at 3 mo of age decreased percentage of heifers that reached puberty by 14 mo of age (*Fig. 3*). Onset of puberty was not significantly altered by ULO. In addition, GRFi delayed puberty in Angus and Simmental heifers. Thus, our failure to delay puberty in Simmental heifers via GRFi at 6 mo of age was due to a breed difference in timing of immunization.

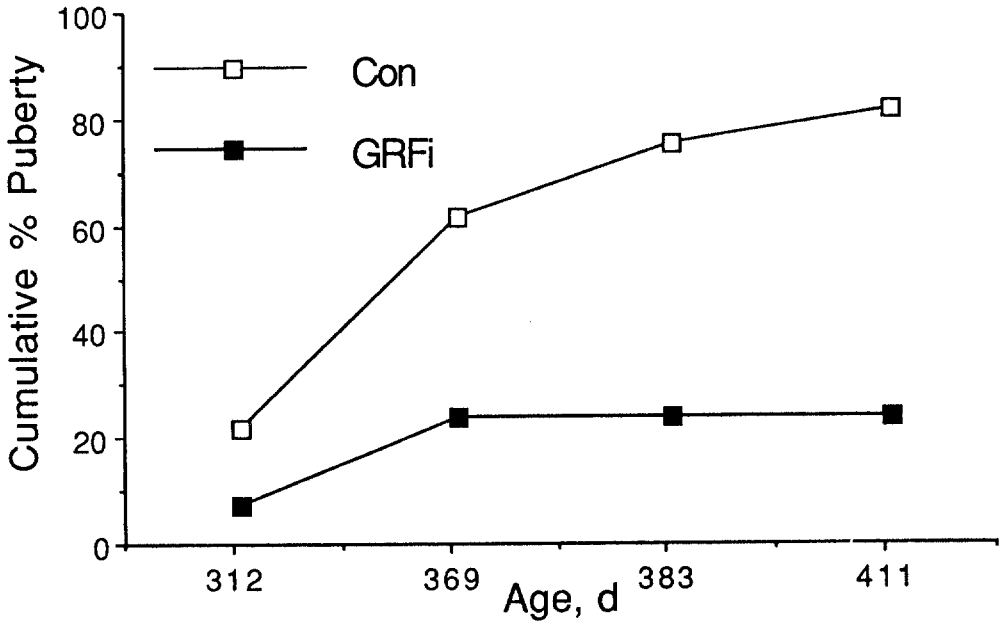

Fig. 3. Attainment of puberty in heifers immunized against GRF or HSA at 3 mo of age, or heifers not treated. GRFi and HSAi heifers were unilaterally ovariectomized at 6 mo of age. Unilateral ovariectomy did not alter timing of puberty, thus data from control and HSAi heifers were pooled (Con). GRFi at 3 mo of age delayed puberty beyond 15 mo in over 75 % of heifers (84).

Summarization of effects of GRFi at 3 mo of age on follicular growth and FFL concentrations of IGF-I and IGFBP at 6 mo of age provided valuable insight as to possible mechanisms through which GRFi delayed puberty (84). Number of large, but not small or medium sized follicles at 6 mo of age were decreased by GRFi (.3 $\pm$.2 vs 1.1 $\pm$.4). In addition, we found that serum and FFL $\leqslant$4 mm follicles) concentrations of IGF-I were lower in GRFi than in HSAi heifers. In addition to lowering concentrations of IGF-I in both compartments, GRFi dramatically reversed the ratio of serum to FFL IGF-I

(HSAi — serum >FFL; GRFi — FFL >serum). *In vitro* reports have demonstrated that IGF-I is synthesized by granulosa cells (35,36); however, we do not know whether decreased FFL IGF-I reflects transudation of serum IGF-I and(or) reduction in ovarian synthesis of IGF-I. A shift in the ratio of serum to FFL IGF-I by GRFi provides indirect evidence for an ovarian site of action.

Equally important to effects of GRFi on serum and FFL IGF-I is potential effects of GRFi on IGFBP. As anticipated (19), GRFi increased serum IGFBP-2 (84). In contrast to IGF-I, FFL ($\leqslant$ 4 mm follicles) concentrations of IGFBP-2 were not significantly effected by GRFi. However, apparent concentrations of FFL IGFBP-3 were decreased by GRFi. IGFBP-3 has been demonstrated *in vitro* to have inhibitory and stimulatory effects on IGF-I action (19). Most effects of IGFBP on IGF-I-mediated FSH action in ovarian cultures have been inhibitory (35,36,85,86). Consistent with this effect is the observation that diabetes increases follicular atresia (31) and 31 kDa IGFBP in swine (32,33). Future studies will be necessary to clearly define the role of IGFBP in mediating the effects of GRFi on puberty in heifers.

Initial experiments have been conducted with the goal of stimulating preovulatory follicular growth and ovulation in acyclic, GRFi heifers. Acyclic, GRFi heifers were administered recombinant ST (sometribove) or vehicle daily for 56 days (82). Ultrasonography of follicles revealed that diameter of largest follicle was increased after 3 days of ST; however, a chronic effect on ovarian function was not detected. Future replacement therapies may need to be initiated at 3 to 6 mo of age to be effective.

Collectively, these studies indicate that follicular growth between 3 and 6 mo of age is particularly sensitive to alterations in serum and(or) ovarian IGF-I. In addition, we speculate that GRFi at 3 mo of age disrupts normal patterns of follicular growth. Waves or cycles of follicular growth have been reported for prepuberal heifers (87,88); therefore, GRFi may delay puberty by altering or blocking waves of follicular growth.

Immuno-neutralization of GRF may decrease follicular growth through effects mediated at the ovary, pituitary and(or) hypothalamus. Certainly further studies are necessary; however, the absence of effects on serum LH in two studies (72,83) provides strong evidence for an ovarian effect. Studies in rats (89) and mice (90) with low serum ST and delayed puberty also provide evidence for gonadal effects of IGF-I. It is of equal importance to elucidate the component(s) of the GRF-ST-IGF-I axis that mediates GRFi effects on ovarian function. The wealth of information with regard to effects of IGF-I on granulosa cell function (35-37) leads to strong speculation that IGF-I is an important mediator. IGF-I may have direct effects at the ovary through paracrine, autocrine or endocrine effects. Somatotropin may be involved, certainly *via* alteration of serum IGF-I, but also *via* effects on the ovary.

Determination of effects of GRFi on tissue (hypothalamus or ovarian) expression of putative signals such as IGF-I , IGFBP-2, -3, -4- and(or) -5 is necessary to begin to elucidate this mechanism.

In conclusion, active immunization against GRF (through associated changes in ST, IGF-I, and(or) insulin) results in aberrations in follicular growth, possibly mediated through alteration in follicular IGF-I and(or) IGFBP. However, a direct effect of GRFi on ovarian GRF cannot be excluded. Clearly, GRFi at 3 mo of age altered follicular growth culminating in delay in normal timing of estrus. Future studies will be required to better elucidate the site through which lowered IGF-I , ST and(or) GRF alter follicular growth, ultimately delaying puberty. These data indicate that follicular growth and associated endocrine events from 3 to 6 mo of age are critically important for normal onset of puberty.

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